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14. ABSTRACT

The loss of stromal TGF-β signaling has been shown to initiate prostate cancer (PCa) and promote PCa progression. A further effect on osteoblastic bone lesion development was hypothesized and tested in this proposal. Using the *Col*^{cre}*/Tgfbr2* KO mice, we are able to knock out TGF-β signaling specifically in the prostate fibroblasts and in bone osteoblasts. We found that PC3 cell osteolytic bone lesions were significantly increased in the KO mice tibiae compared to the flox mice tibia. bFGF was the only cytokine up-regulated (among many others down-regulated) in KO/PC3 tibiae relative to Flox/PC3 tibiae. However, osteoblastic bone lesions induced by LUCaP cells were inhibited in KO mice tibiae relative to Flox mice tibia in our preliminary study. Our findings suggest that osteoblastic TGF-β signaling inhibits PCa osteolytic bone lesions but may promote PCa osteoblastic bone lesions.

15. SUBJECT TERMS
TGF-β signaling, osteoblasts, fibroblasts, osteolytic, osteoblastic, bone lesions, cytokines, bFGF

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Introduction: The hypothesis of this proposal is that cytokines/chemokines regulated by stromal TGF-β signaling from the primary tumor microenvironment dictate prostate cancer (PCa) osteoblastic bone metastasis. We proposed to determine the contribution of prostate mesenchymal TGF-β in PCa-induced osteoblastic bone lesion development and to determine the chemokines that induced by loss of TGF-β signaling mediate PCa blastic bone metastasis. This grant was transferred on Oct. 9, 2013 from Vanderbilt University to Van Andel Research Institute, where I took a faculty position as an Assistant Professor. The proposed work started by generating TGF-β type II receptor (TβRII) knock-out mice (*Tgfbr2* KO) and TGF-β type I constitutively active transgenic mice. All these mice have been successfully rederived in the VARI facility for breeding. After we started our *in vivo* mouse experiments, which require three survival surgeries, we found that the majority of the mice died followed the third surgery. We are now trying alternative approaches for the *in vivo* studies, and the results are expected in the next two months.

Meanwhile, we have broadened our research scope using the same animal models, investigating the role of mesenchymal TGF-β in the bone microenvironment on PCa-induced bone lesion development. We have found that TGF-β signaling in osteoblasts inhibited osteolytic bone lesion development by PC3 prostate cancer cells, and several cytokines, such as basic fibroblast growth factor (bFGF), have been identified as potential mediators. Preliminary experiments have found that TGF-β signaling in osteoblasts promoted osteoblastic bone lesion development by the LUCaP PCa cells.

Keywords: TGF-β signaling, osteoblasts, fibroblasts, osteolytic, osteoblastic, bone lesions, cytokines/chemokines, bFGF

Overall Project Summary:

Task 1 (months 1-16) was "to investigate the effect of prostate mesenchymal TGF-β responsiveness in PCa osteoblastic bone lesion development." We have successfully accomplished **Task 1a**, "breeding of $Col^{creERT}/Tgfbr1^{T204D}$ (Tgfb-On) and $Col^{creERT}/Tgfbr2^{floxE2/floxE2}$ (Tgfb-Off) mice and their respective control mice for initial experiments". These mice were crossed with mT/mG reporter mouse from Jackson Laboratory. The specific Cre expression can be visualized directly using green fluorescent protein (GFP) in mouse prostate and cultured prostate fibroblasts (**Figure 1**) or through immunohistochemistry (IHC) using anti-GFP antibody in decalcified mouse bone tissues (**Figure 2**). The specific Col^{Cre} expression in fibroblasts of the prostate and osteoblasts of the bone was confirmed. We also performed **Tasks 1b** and **1c**, according to our initial experiment plan (**Figure 3**). However, because so many mice died after three survival surgeries, we are now modifying our procedure to the alternative one, shown in **Figure 4**. In about two months, we will be able to perform **Task 1c**, "to establish bone lesion development model using the orthotopic grown tumor cells" and **Task 1d,** "IHC of harvest tissues and data analysis".

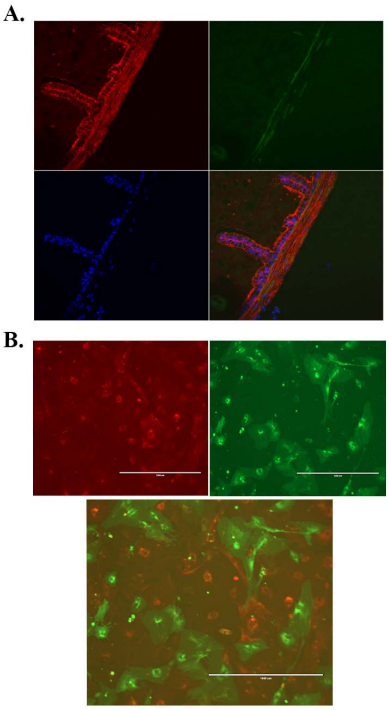


Figure 1. Fibroblast specific Cre expression in Col^{creERT} mouse prostate (A), and in the cultured prostate fibroblasts (B). The Cre positive cells are green.

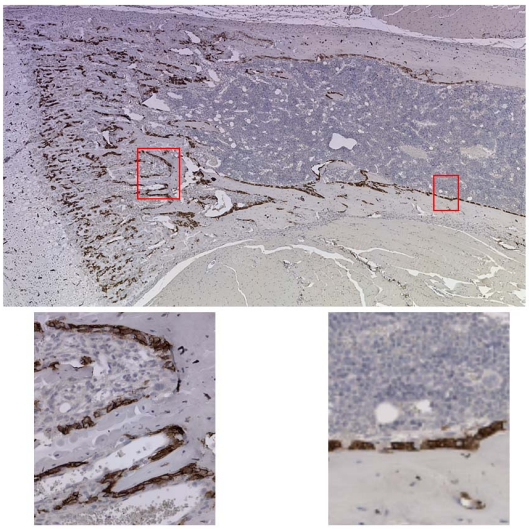


Figure 2. IHC identified the specific cre expression in the osteoblasts of the bone in col^{creERT} mouse.

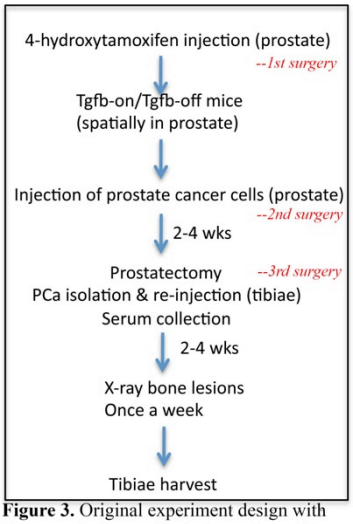


Figure 3. Original experiment design with three survival surgeries to be performed in one mouse.

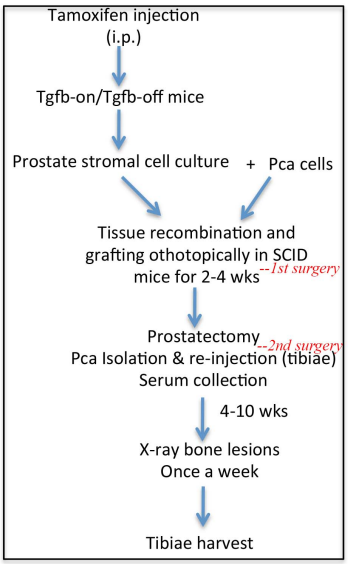


Figure 4. Modified experimental design. A total of two survival surgeries will be performed in one mouse.

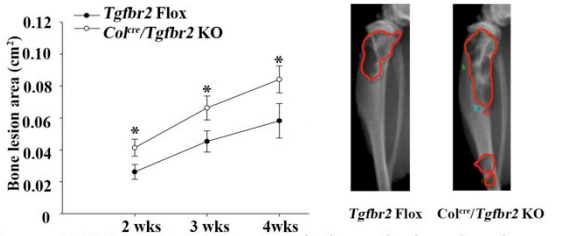
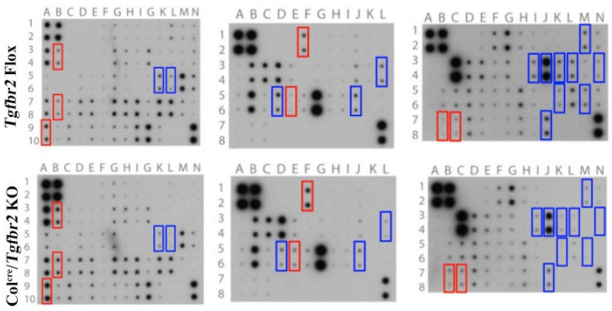


Figure 5. PC3 induced osteolytic bone lesion developmen in the *Tgfbr2* Flox or *col*^{cre}*/Tgfbr2* KO mice. Bone lesions were monitored by weekly X-ray from 2 to 4 weeks post injection. Lytic lesion areas were measured on the X-ray images using Metmorph software. Reprentative image of 4wks' bone lesions were shown. *n=9* mice at least for each time point. *p<0.5 by Student's *t* test, two-tailed.



Increased (KO/Flox)	**Fas Ligand, MCP-1, SDF-1a, bFGF, Resistin, MFG-E8, Neprilysin,**
Decreased (KO/Flox)	Leptin, Leptin receptor, Pro-MMP-9, TROY, MMP-2, Epiregulin, Galectine-1, Growth arrest specific 1, Growth arrest specific 6, CD36, Granzyme B, IL-21, IL-6R, TWEAK R

Figure 6. Different cytokine expression between *Tgfbr2* Flox and Col^{cre}/*Tgfbr2* KO mice tibiae after 3wks' post PC3 tibial injection. Cytokine array were performed using the total protein extractions from the PC3 injected tibae of the *Tgfbr2* Flox and Col^{cre}/*Tgfbr2* KO mice. The increased and decreased cytokines were identified and listed.

The scope of this proposal was expanded logically due to our findings of the loss of stromal TβRII in the PCa bone metastatic tissues (Li, 2012). We thus investigated the stromal TGF-β effect on PCa bone lesion development. We found that PC3 PCa-induced osteolytic bone lesion development was promoted in the *Col^{cre}/Tgfbr2* KO mice relative to control *Tgfbr2 flox* mice (**Figure 5**). Further, the differences in expression of chemokines between the PC3-induced bones from KO and flox mice were compared using cytokine array analysis (**Figure 6**). Basic fibroblast growth factor (bFGF) was the only factor to have increased expression at both mRNA level and protein level in the PC3/KO tibiae relative to the PC3/flox tibiae (**Figure 7**). We are now investigating the function of bFGF in the osteoblastic TGF-β signaling effect on PCa bone lesion development. In contrast to PCa osteolytic lesions, our initial experiment using LUCaP prostate tumor cells revealed that PCa-induced osteoblastic bone lesion development was inhibited in the *Col^{cre}/Tgfbr2* KO mice relative to the control *Tgfbr2 flox* mice (**Figure 8**).

The osteoblastic TGF-β effect on PCa bone lesion development was also investigated in the $Col^{creERT}/Tgfbr1^{T204D}$ mice using the same strategy. No significant difference was found in the Tgfb-On mice relative to controls. Considering this is a transgenic, but not a knock-in, mouse model, we will focus on the Tgfb-Off knock-out mouse line in future experiments.

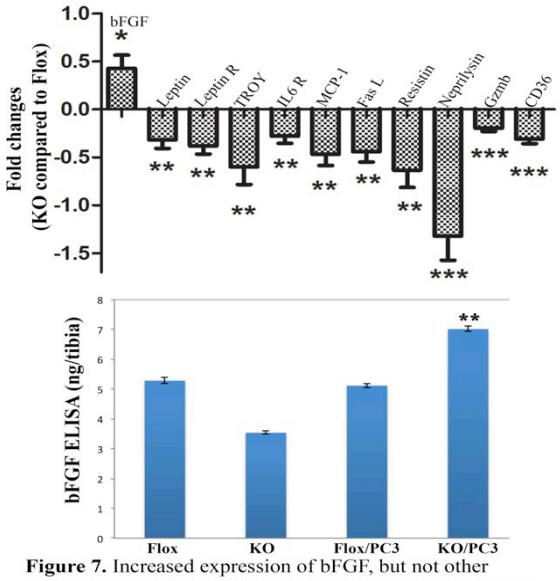


Figure 7. Increased expression of bFGF, but not other cytokines, were detected in the PC3 induced tibiae from the Col^{cre}/Tgfbr2 KO mice compared to those from the Tgfbr2 Flox mice, at both mRNA and protein levels by qRT-PCR and ELISA respectively. n=4 at least of each group. *<0.05, **<0.001, ***<0.0001 by student t test, two-tailed.

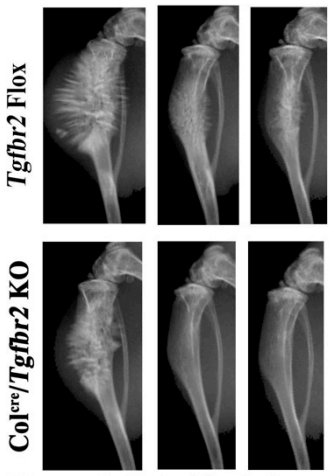


Figure 8. Osteoblastic bone lesions by LUCaP cells post 12 wks' injection in the tibiae of the *Tgfbr2* Flox or Col^{cre}/*Tgfbr2* KO mice.

Key Research Accomplishments:

1. We bred and characterized the tamoxifen-inducible fibroblasts and osteoblasts in Tgfb-On and Tgfb-Off mice.
2. We discovered that TGF-β signaling in the osteoblasts inhibited PC3 PCa-induced osteolytic bone lesion development.
3. We identified increased bFGF expression as a potential downstream mediator of the effect of osteoblastic TGF-β signaling on PCa-induced osteolytic bone lesion development.
4. We have preliminary findings that TGF-β signaling in the osteoblasts may promote LUCaP cell osteoblastic bone lesions.

Conclusions:

1. This sponsored research is ongoing as proposed with minor modifications.
2. Cytokines such as bFGF, mediated by TGF-β signaling in the osteoblasts, inhibit PCa-induced osteolytic bone lesion development.

Abstracts and Presentations:

2014 AACR abstract, #4839

TGF-β signaling in osteoclasts promotes, but in osteoblasts inhibits, prostate cancer-induced bone lesions

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[Q: I did not edit the abstract except for a few typo corrections.]

Bone is the only clinically detected metastatic site in advanced prostate cancer (PCa) patients. Bone metastases were found in 70% of patients who died of PCa. The mechanism of PCa bone metastasis is largely unknown, partially due to different types of bone lesions existing in the

same patients. Transforming growth factor beta (TGF-β) is known to be abundant in the bone microenvironment and a key factor driving cancer cell colonization and proliferation in the bone, and inducing the bone lesion development. Previous studies have shown that blocking the pathway systemically or in cancer cells reduces the breast cancer and melanoma bone metastases in animal models, presenting an exciting target for cancer therapy. However, TGF-β is also directly affecting the proliferation and differentiation of all the cells in the bone microenvironment. Blocking TGF-β in bone metastases patients will only be possible until the cell specific contribution of TGF-β signaling in the bone microenvironment to cancer cells were further delineated. We hypothesized that cell specific TGF-β signaling in the bone microenvironment has a distinct role in PCa bone metastasis.

Genetically engineered mouse (GEM) models were used to delineate the role of mesenchymal cell- or myeloid cell-specific TGF-β signaling on PCa-induced osteolytic bone lesion development. Lysozyme M promoter-driven Cre was used to induce the ablation of the TGF-β type II receptor (*Tgfbr2*) in mature macrophages, granulocytes and osteoclasts, thus to knockout TGF-β signaling in these cells [$LysM^{cre}$/$Tgfbr2^{floxE2/floxE2}$/Rosa26/$Rag2^{-/-}$ (Tgfb_off_OC)]. The collagen promoter-driven Cre was used to knock out TGF-β signaling in fibroblasts, chondrocytes and osteoblasts [Col^{creERT}/$Tgfbr2^{floxE2/floxE2}$/Rosa26/$Rag2^{-/-}$ (Tgfb_off_OB)]. PC3 PCa cells were injected into the tibiae of the GEMs and their respective Cre-controlled mice. The host mice tibiae were imaged using Faxitron x-ray every week from 2 to 4 weeks post tumor injection; the bone lesion areas were measured and analyzed by Metamorph.

We found that the osteolytic bone lesion development was significantly reduced in the Tgfb_off_OC mice compared to the control mice at 4 weeks post tumor inoculation ($p<0.05$). In contrast, PC3 cells in the Tgfb_off_OB mice compared to the control mice significantly promoted osteolytic bone lesion development started from 2 weeks and up to 4 weeks post tumor injections ($p<0.05$). All statistics were applied by Student's t tests. These results suggest that TGF-β signaling activation is anti-osteolytic in osteoblasts, but pro-osteolytic in osteoclasts in this PC3 induced bone lysis models.

2014 AACR poster presentation, poster attached.

Reportable outcomes:

1. 2014 AACR abstract and poster presentation, #4839.
2. 2014 AACR-Prostate Cancer Foundation Scholar-in-Training Award.

References:

Li X, Sterling JA, Fan KH, Vessella RL, Shyr Y, Hayward SW, Matrisian LM, Bhowmick NA, 2012. Loss of TGF-beta responsiveness in prostate stromal cells alters chemokine levels and facilitates the development of mixed osteoblastic/osteolytic bone lesions. *Mol Cancer Res,* 10:494-503. http://www.ncbi.nlm.nih.gov/pubmed/22290877

Appendices

AACR poster

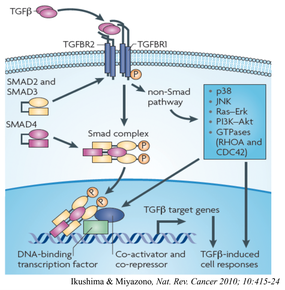
TGF-β signaling in osteoclasts promotes, but in osteoblasts inhibits prostate cancer induced bone lesions

Xiangqi Meng[1], Priscilla Lee[1], Neil A. Bhowmick[2], Xiaohong Li[1]*

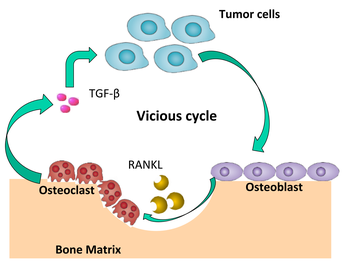
[1]Center for Skeletal Disease and Tumor Metastasis, Van Andel Institute, Grand Rapids, MI [2]Samuel Oschin Comprehensive Cancer Institute and Department of Medicine, Cedars Sinai Medical Center, Los Angeles, CA

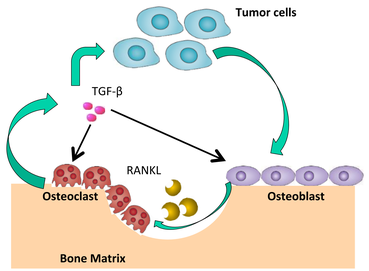
Background

- Prostate cancer(Pca) bone metastases are found in 70% of patients who died of Pca.
- Transforming growth factor beta (TGF-β) is known to be the most abundant growth factor in the bone microenvironment, which is a key factor driving cancer cell colonization and proliferation in the bone.
- Although studies have shown that blocking the pathway systemically or in cancer cells reduces the breast cancer and melanoma bone metastases in animal models, presenting an exciting target for cancer therapy. However, the benefit of blocking TGF-β signaling in clinical trials is not conclusive.



Ikushima & Miyazono, *Nat. Rev. Cancer 2010; 10:415-24*





Gap in knowledge

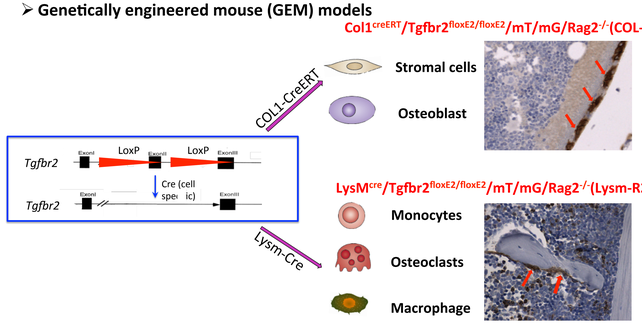
- The cell specific role of TGF-β signaling in the bone microenvironment on Pca bone metastasis has not been fully understood.

Hypothesis

- **Cell specific TGF-β signaling in the bone microenvironment has distinct effects on Pca bone metastasis.**

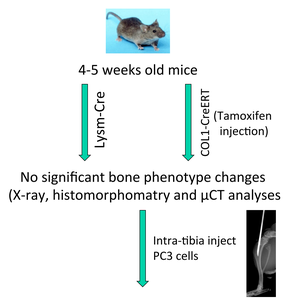
Approaches

- **Genetically engineered mouse (GEM) models**



Based on the Cre/Loxp system, we developed two specific Cre expression mice models—LysM^{cre}/Tgfbr2$^{floxE2/floxE2}$/mT/mG/Rag2$^{-/-}$ (Lysm-Cre) and Col1^{creERT}/Tgfbr2$^{floxE2/floxE2}$/mT/mG/Rag2$^{-/-}$(COL1-Cre). The Lysm-Cre is specifically expressed in the myeloid cell lineage(including osteoclast), and the COL1-Cre is specifically expressed in the mesenchyme lineage(including osteoblast). Immunohistochemistry showed the Cre expression cells in bone microenvironment. (GFP staining, 1:250)

- **Experimental Design**



Results

- **Blocking TGF-β signaling in osteoclasts inhibited osteolytic bone lesion development**

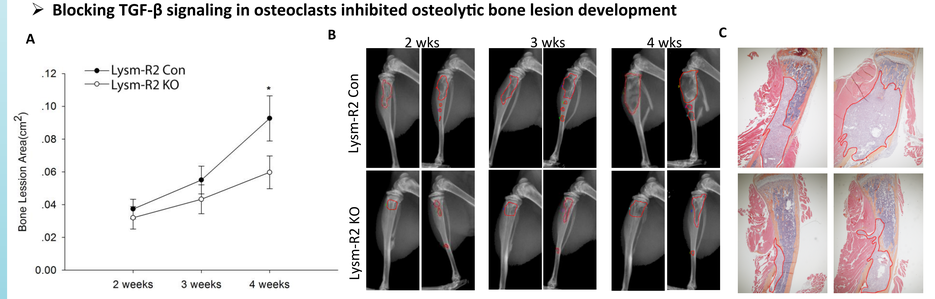


Figure 1. Loss of TGF-β signaling in osteoclasts inhibits PC3 induced osteolytic bone lesion development. **A**:Osteolytic bone lesion area induced by PC3 cells were significantly decreased in the Lysm-R2 KO mice compared to control mice (n=14 at least; P<0.01).**B**: Representative X-ray images of the lytic bone lesions at each time point. The circled regions showed the lytic bone lesion areas that were measured for statistic analysis. **C**:Representative H&E histology of the tibiae of 4 wks' post PC3 injection, which confirmed our X-ray data and Pca growth in the bone marrow.

- **Blocking TGF-β signaling in osteoblasts promoted osteolytic bone lesion development**

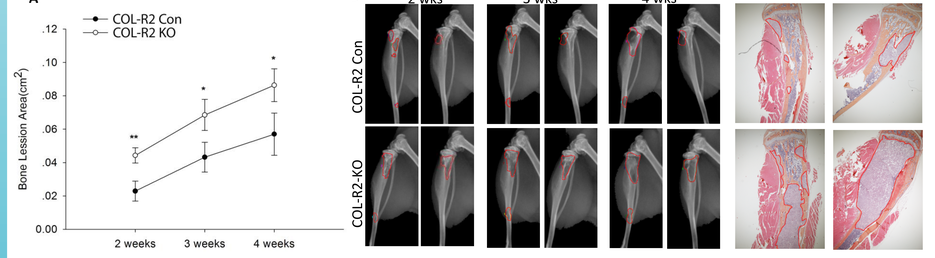


Figure 2. Loss of TGF-β signaling in osteoblasts promotes PC3 induced osteolytic bone lesion development. **A**:Osteolytic bone lesion area induced by PC3 cells were significantly increased in the COL1-R2 KO mice compared to control mice (n=9 at least; P<0.05).**B**: Representative X-ray images of the lytic bone lesions at each time point. The circled regions showed the lytic bone lesion areas that were measured for statistic analysis. **C**:Representative H&E histology of the tibiae of 4 wks' post PC3 injection, which confirmed our X-ray data and Pca growth in the bone marrow.

- **Blocking TGF-β signaling in osteoblasts changed the cytokines expression profiles in the contact of PC3 prostate cancer cells**

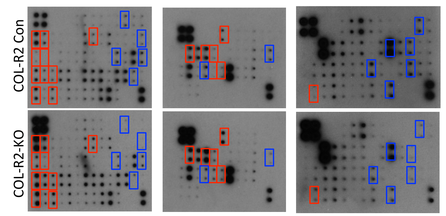


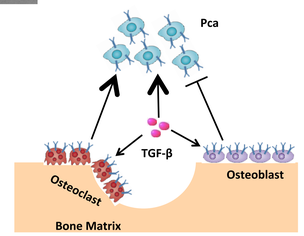
Figure 3. Cytokine array data revealed that certain cytokines were dysregulated in COL-R2 KO mice compared to COL-R2 Con mice. Analysis was conducted with COL-R2 KO and COL-R2 Con mice from at least 3 different mice per group. The red circled box indicated the unregulated cytokines compared to the COL-R2 Con, and the blue ones indicated the downregulated ones.

Table 1. List of dysregulated cytokines in the COL-R2-KO mice compared to the COL-R2-Control mice

KO/Con	Cytokines
Up	IL-4,IL-5,SDF-1a,Lymphotactin,MCP1,TCA-3,MCP-5, IGBP-5, Fas ligand, Eotaxin-2, bFGF, Shh-N, Resistin, IGF-1, IGF-II, ICAM-1, MFG-E8
Down	Leptin R, RANTES, L-selectin, CTACK, IL-3 Rb, Pro-MMP-9, MMP-2, Il-28, Growth arrest specific 6, TWEAK R, IL-1ra/IL-1F3, CD36, Galectin-1

Summary and Conclusion

Targeted blocking of TGF-β signaling in specific cell types in the bone microenvironment should be applied for Pca bone metastasis therapy.



Achknowledgement

AACR-Prostate Cancer Foundation Scholar-in-Training Award (X. Meng)

DOD W81XWH-12-1-0271 (X. Li), VAI Start-up Fund 53010A (X. Li)